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(54) Title: POLYPEPTIDES HAVING A SINGLE COVALENTLY BOUND N-TERMINAL WATER-SOLUBLE POLYMER

## (57) Abstract

This invention provides compositions consisting essentially of a polypeptide and a water-soluble polymer covalently bound thereto at the N-terminal  $\alpha$ -carbon atom via a hydrazone or reduced hydrazone bond, or an oxime or reduced oxime bond. This invention also provides methods of making the instant compositions, pharmaceutical compositions comprising same, and kits for use in preparing same.

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POLYPEPTIDES HAVING A SINGLE COVALENTLY BOUND N-TERMINAL  
WATER-SOLUBLE POLYMER

5

Field of the Invention

The instant invention relates to polypeptides which  
10 have bound at their N-termini a single, water soluble  
polymer. These polypeptides have properties which render  
them advantageous for use as pharmaceutical and diagnostic  
agents. The invention also relates to methods of making  
these polypeptides, and related pharmaceutical  
15 compositions and kits.

Background of the Invention

20

Throughout this application, various publications are  
cited. The disclosure of these publications is hereby  
incorporated by reference into this application to  
describe more fully the state of the art to which this  
25 invention pertains.

In recent years, non-antigenic water-soluble  
polymers, such as polyethylene glycol ("PEG"), have been  
used for the covalent modification of polypeptides of  
30 therapeutic and diagnostic importance. For example,  
covalent attachment of PEG to therapeutic polypeptides  
such as interleukins (Knauf, M. J. et al., *J. Biol.  
Chem.* 1988, 263, 15,064; Tsutsumi, Y. et al., *J.  
Controlled Release* 1995, 33, 447), interferons (Kita, Y.  
35 et al., *Drug Des. Delivery* 1990, 6, 157), catalase

(Abuchowski, A. et al., *J. Biol. Chem.* 1977, 252, 3,582), superoxide dismutase (Beauchamp, C. O. et al., *Anal. Biochem.* 1983, 131, 25), and adenosine deaminase (Chen, R. et al., *Biochim. Biophys. Acta* 1981, 660, 293),  
5 has been reported to extend their half life *in vivo*, and/or reduce their immunogenicity and antigenicity.

However, such methods have serious drawbacks. Specifically, in most instances, PEG molecules are  
10 attached through amino groups on polypeptides using methoxylated PEG ("mPEG") having different reactive moieties. Such polymers include mPEG-succinimidyl succinate, mPEG-succinimidyl carbonate, mPEG-imidate, and mPEG-cyanuric chloride. The attachment using these  
15 polymers was usually non-specific, i.e., occurring at various amino groups on the polypeptides in a random fashion, and not exclusively at a particular amino group. Such non-specific attachment may modify amino acid residues at active sites in such a manner as to  
20 eliminate the biological activity of the polypeptides. Also, the resultant conjugates may contain a heterogeneous mixture of modified polypeptide, which is undesirable for pharmaceutical use.

25 To overcome these problems, it was desirable to site-specifically attach a polymer to a polypeptide. For the polypeptide, doing so would preserve biological activity, prolong blood circulating time, reduce immunogenicity, increase aqueous solubility, and enhance  
30 resistance to protease digestion. Site-specific pegylation at the N-terminus, side chain and C-terminus

of a potent analog of growth hormone-releasing factor has been performed through solid-phase synthesis (Felix, A. M. et al., *Int. J. Peptide Protein Res.* 1995, 46, 253). Since the specific pegylation was accomplished 5 during assembly of the peptide on a resin, the method can not be applied to an existing peptide.

An additional method used involved attaching a peptide to extremities of liposomal surface-grafted PEG 10 chains in a site-specific manner through a reactive aldehyde group at the N-terminus generated by sodium periodate oxidation of N-terminal threonine (Zalipsky, S. et al., *Bioconj. Chem.* 1995, 6, 705). However, this method is limited to polypeptides with N-terminal serine 15 or threonine residues.

Enzyme-assisted methods for introducing activated groups specifically at the C-terminus of a polypeptide have also been described (Schwarz, A. et al., *Methods Enzymol.* 1990, 184, 160; Rose, K. et al., *Bioconjugate Chem.* 1991, 2, 154; Gaertner, H. F. et al., *J. Biol. Chem.* 1994, 269, 7224). Typically, these active groups can be hydrazide, aldehyde, and aromatic-amino groups for subsequent attachment of functional probes to 20 polypeptides. However, since the methods are based on the specificity of proteases, they require extreme caution, and the scope of their application is limited. 25

Site-specific mutagenesis is a further approach 30 which has been used to prepare polypeptides for site-specific polymer attachment. WO 90/12874 describes the

site-directed pegylation of proteins modified by the insertion of cysteine residues or the substitution of other residues for cysteine residues. This publication also describes the preparation of mPEG-erythropoietin 5 ("mPEG-EPO") by reacting a cysteine-specific mPEG derivative with a recombinantly introduced cysteine residue on EPO. Similarly, interleukin-2 was pegylated at its glycosylation site after site-directed mutagenesis (Goodson, R. J. et al., *Bio/Technology* 1990, 10 8, 343).

Glycoproteins provide carbohydrates as additional target sites for modification. The enzyme peroxidase has been modified with PEG-diamine through its carbohydrate 15 moiety (Urrutigoity, M. et al., *Biocatalysis* 1989, 2, 145). WO 94/28024 describes the methods for preparing mPEG-EPO through periodate-oxidized carbohydrate. The chemistry involved was hydrazone formation by reacting mPEG-hydrazide with aldehyde groups of the carbohydrate 20 moiety on EPO. This type of modification generates reactive aldehyde groups through an oxidation step, which potentially can oxidize various types of sugar residues in the carbohydrate moiety and some amino acid residues in the polypeptide, such as methionine. Another 25 disadvantage of this method stems from the heterogeneity of the carbohydrate moieties of EPO. EPO expressed from Chinese hamster ovary cells has four carbohydrate chains, which include three N-linked chains at asparagines 24, 38, and 83 and one O-linked chain at 30 serine 126. A total of 52 different N-linked, and at least 6 O-linked, oligosaccharide structures have been

identified (Rush, R. S. et al., *Anal. Chem.* 1995, 67, 1442; Linsley, K. B. et al., *Anal. Biochem.* 1994, 219, 207). Accordingly, it is difficult to control the number of, or attachment sites of, polymer molecules when 5 modifying EPO or other protein via its carbohydrate chains.

In short, the methods in the art for attaching a water-soluble polymer to a polypeptide suffer from serious 10 drawbacks. These drawbacks include the following: (a) a lack of precision, both stoichiometrically and with respect to the situs of attachment; (b) the need to perform difficult and labor-intensive techniques such as site-specific mutagenesis; (c) the need to use solid-phase 15 peptide synthesis concurrently with polymer attachment, instead of attaching a polymer to a pre-existing polypeptide; and (d) the rigid requirement that the identity of the N-terminal amino acid residue be threonine or serine.

20

For some time, there has existed a need for a general method of site-specifically attaching a water-soluble polymer to the N-terminal amino acid residue of a polypeptide, which method does not suffer from the above- 25 identified drawbacks. However, no such method exists.

Summary of the Invention

5 This invention provides two compositions of matter. The first composition of matter consists essentially of a polypeptide and a water-soluble polymer covalently bound thereto at the N-terminal  $\alpha$ -carbon atom of the polypeptide via a hydrazone bond or reduced hydrazone bond, with the  
10 proviso that (a) the polymer has a molecular weight of from about 200 to about 200,000 daltons, (b) the natural function of the polypeptide is not eliminated upon removal of its N-terminal  $\alpha$ -amino group, and (c) the polypeptide's N-terminal amino acid residue is not serine or threonine.

15 The second composition of matter consists essentially of a polypeptide and a water-soluble polymer covalently bound thereto at the N-terminal  $\alpha$ -carbon atom of the polypeptide via an oxime bond or reduced oxime bond, with  
20 the proviso that (a) the polymer has a molecular weight of from about 200 to about 200,000 daltons, and (b) the natural function of the polypeptide is not eliminated upon removal of its N-terminal  $\alpha$ -amino group.

25 This invention also provides four methods of covalently binding a water-soluble polymer to the N-terminal  $\alpha$ -carbon atom of a polypeptide. The first method, which binds the polymer to the carbon atom via a hydrazone bond, comprises the steps of  
30 (a) contacting the polypeptide with (i) glyoxylate ion or derivative thereof at a concentration of from about 0.1 M to about 2.0 M, (ii) a transition metal ion at

a concentration of from about 10  $\mu\text{M}$  to about 1  $\text{M}$ , and  
5 (iii) a Lewis base at a concentration of from about 10  $\text{mM}$  to about 10  $\text{M}$ , at a pH of from about 3.0 to about 8.0 and a temperature of from about 0°C to about 100°C, so as to form a transaminated polypeptide having an N-terminal  $\alpha$ -carbonyl group; and

10 (b) contacting the transaminated polypeptide, at a pH of from about 1.0 to about 7.5, with a water-soluble polymer having a moiety covalently bound thereto which reacts with the transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form a hydrazone bond, thereby covalently binding the polymer to the N-terminal  $\alpha$ -carbon atom of the polypeptide via a hydrazone bond, with the proviso that the polymer has a molecular weight of from about 200 to about 200,000 daltons, and the natural function of the polypeptide is not eliminated upon removal of its N-terminal  $\alpha$ -amino group.

20

The second method, which binds the polymer to the carbon atom via an oxime bond, comprises the steps of

25 (a) contacting the polypeptide with (i) glyoxylate ion or derivative thereof at a concentration of from about 0.1  $\text{M}$  to about 2.0  $\text{M}$ , (ii) a transition metal ion at a concentration of from about 10  $\mu\text{M}$  to about 1  $\text{M}$ , and (iii) a Lewis base at a concentration of from about 10  $\text{mM}$  to about 10  $\text{M}$ , at a pH of from about 3.0 to about 8.0 and a temperature of from about 0°C to about 100°C, so as to form a transaminated

polypeptide having an N-terminal  $\alpha$ -carbonyl group;  
and

(b) contacting the transaminated polypeptide, at a pH of  
from about 1.0 to about 7.5, with a water-soluble  
5 polymer having a moiety covalently bound thereto  
which reacts with the transaminated polypeptide's N-  
terminal  $\alpha$ -carbonyl group to form an oxime bond,  
thereby covalently binding the polymer to the N-  
terminal  $\alpha$ -carbon atom of the polypeptide via an  
10 oxime bond, with the proviso that the polymer has a  
molecular weight of from about 200 to about 200,000  
daltons, and the natural function of the polypeptide  
is not eliminated upon removal of its N-terminal  $\alpha$ -  
amino group.

15

The third method comprises the steps of the first  
method, as well as a further step of reducing the  
hydrazone bond formed in step (b). The fourth method  
comprises the steps of the second method, as well as a  
20 further step of reducing the oxime bond formed in step  
(b).

This invention also provides a pharmaceutical  
composition which comprises an effective amount of the  
25 instant first or second composition, and a  
pharmaceutically acceptable carrier.

Finally, this invention provides kits for use in  
preparing the instant compositions. The first kit, for  
30 preparing the first instant composition, comprises the  
following:

(a) a glyoxylate ion or derivative thereof;  
(b) a transition metal ion;  
(c) a Lewis base; and  
5 (d) a water-soluble polymer having a molecular weight of from about 200 to about 200,000 daltons, and having a moiety covalently bound thereto which reacts with a transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form a hydrazone bond, thereby covalently binding the polymer to  
10 the N-terminal  $\alpha$ -carbon atom of the polypeptide.

The second kit, for use in preparing the second instant composition, comprises the following:

(a) a glyoxylate ion or derivative thereof;  
15 (b) a transition metal ion;  
(c) a Lewis base; and  
16 (d) a water-soluble polymer having a molecular weight of from about 200 to about 200,000 daltons, and having a moiety covalently bound thereto which reacts with a transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form an oxime bond,  
20 thereby covalently binding the polymer to the N-terminal  $\alpha$ -carbon atom of the polypeptide.

Brief Description of the Figures

Figure 1 shows a scheme for preparing N-terminal modified EPO. This is a two-step reaction:  
5 transamination and pegylation. Four mPEG5000 (i.e., mPEG having a m.w. of 5000 daltons) derivatives with hydrazine carboxylate (HZC), hydrazide (HZ), semicarbazide (SCZ) and oxylamine functional groups were used.

10

Figure 2 shows the gel filtration chromatogram of mPEG-EPO with a hydrazone bond formed using a hydrazine carboxylate (HZC) moiety, native EPO, and mPEG5000 hydrazine carboxylate on a TSK G3000SW<sub>XL</sub> column (7.5 x 30 mm). The mobile phase is 20 mM sodium citrate (pH 7.0) containing 100 mM NaCl.  
15

Figure 3 shows a matrix-assisted laser desorption time-of-flight mass spectra of mPEG5000 hydrazine carboxylate, native EPO, and mPEG-EPO with a hydrazone bond formed using a hydrazine carboxylate (HZC) moiety.  
20

25

Figure 4 shows the characterization of mPEG-EPO by electrophoresis methods: (1) 4-15% SDS-PAGE, Coomassie stain; (2) Western blot; (3) 4-15% SDS-PAGE, iodine stain; and (4) Isoelectric focusing (IEF, pH 3-7). Lane 1, MW or pI markers; Lane 2, native EPO; Lane 3, Transaminated EPO; and Lanes 4 and 5, mPEG-EPO  
30

with hydrazone bonds formed using hydrazine carboxylate (HZC) and hydrazide (HZ) moieties, respectively.

5 Figure 5 shows a graph of results of an ELISA assay for mPEG-EPO with hydrazone bonds formed using hydrazine carboxylate (HZC) and hydrazide (HZ) moieties.

10 Figure 6 shows a graph of the results of a cell proliferation assay of native EPO, transaminated EPO, and mPEG-EPO with hydrazone bonds formed using hydrazine carboxylate (HZC) and hydrazide (HZ) moieties.

15 Figure 7 shows a graph of the results of an exhypoxic mouse bioassay for mPEG-EPO with hydrazone bonds formed using hydrazine carboxylate (HZC), hydrazide (HZ) and semicarbazide (SCZ) moieties.  
20

Detailed Description of the Invention

This invention provides two compositions of matter. The first composition of matter consists essentially of a 5 polypeptide and a water-soluble polymer covalently bound thereto at the N-terminal  $\alpha$ -carbon atom of the polypeptide via a hydrazone bond or reduced hydrazone bond, with the proviso that (a) the polymer has a molecular weight of from about 200 to about 200,000 daltons, (b) the natural 10 function of the polypeptide is not eliminated upon removal of its N-terminal  $\alpha$ -amino group, and (c) the polypeptide's N-terminal amino acid residue is not serine or threonine.

The second composition of matter consists essentially 15 of a polypeptide and a water-soluble polymer covalently bound thereto at the N-terminal  $\alpha$ -carbon atom of the polypeptide via an oxime bond or reduced oxime bond, with the proviso that (a) the polymer has a molecular weight of from about 200 to about 200,000 daltons, and (b) the 20 natural function of the polypeptide is not eliminated upon removal of its N-terminal  $\alpha$ -amino group.

As used herein, "polypeptide" includes both peptides and proteins. "Peptide" means a polypeptide of fewer than 25 10 amino acid residues in length, and "protein" means a polypeptide of 10 or more amino acid residues in length. In this invention, the polypeptides may be naturally occurring or recombinant (i.e. produced via recombinant DNA technology), and may contain mutations (e.g. point, 30 insertion and deletion mutations) as well as other covalent modifications (e.g. glycosylation and labeling

[via biotin, streptavidin, fluoracine, and radioisotopes such as I<sup>131</sup>). Moreover, each instant composition may contain more than a single polypeptide, i.e., each may be a monomer (one polypeptide bound to a polymer) or a 5 multimer (two or more polypeptides bound to a polymer or to each other).

Polypeptides include, by way of example, monoclonal and polyclonal antibodies, cytokines such as M-CSF and 10 GM-CSF, lymphokines, IL-2, IL-3, growth factors such as PDGF and EGF, peptide hormones such as hGH, EPO and derivatives thereof, blood clotting factors such as Factor VIII, immunogens, enzymes, enzyme inhibitors, and other ligands. In the preferred embodiment of the instant 15 compositions, the polypeptide is EPO or derivative thereof. The EPO may be naturally occurring or recombinant. Derivatives of EPO include, but are not limited to, the polypeptides

GGLYLCRFGPVTWDCGYKGG,  
20 GGTYSCHFGPLTWVCKPQGG,  
GGDYHCRMGPLTWVCKPLGG,  
VGNYMCHFGPITWVCRPGGG,  
GGVYACRMGPITWVCSPLGG,  
VGNYMAHMGPITWVCRPGG,  
25 GGTYSCHFGPLTWVCKPQ,  
GGLYACHMGPMTWVCQPLRG,  
TIAQYICYMGPETWECRSPSKA,  
YSCHFGPLTWVCK, and  
YCHFGPLTWVC,  
30 as well as the mutants listed in Table 1 below.

Table 1

	<u>Mutation</u>	<u>Activity relative to wt*</u>	<u>Reference</u>
	L5S	+/-	9
10	L5S/W51S/M54S/V82S/W88S/ L112A/A124S/A125S	+/-	9
	S9A	++++	3
	R10A	++++	3
	E13A	++++	3
15	R14L	++++	3
	R14A	++	3
	L17A	++++	3
	E18A	++++	3
	K20A	++++	3
20	E21A	++++	3
	N24Q	++	1
	N24Q/N83Q	+	1
	N24Q/N38Q/ N83Q	+++	1
	C29Y/C33Y	++++	3
25	A30S/L35S	+++	9
	A30S/A124S/A125S	++	9
	C33P/R139C	++++	7
	L35S/A124S/A125S	++	9
	N38Q	++	1
30	N38Q/N83Q	++++	1
	V41S	+/-	9
	K45A	++++	3
	F48S	++++	3
	Y49S	++++	3
35	A50S	++++	3
	W51S	++++	3
	W51S/V144S	+	9
	W51S/V82S/W88S	++	9
	W51S/V82S/W88S/V144N	++	9
40	W51S/V82S/W88S/L112A/I119A A124S/A125S	+++	9
	W51S/M54S/V82S/W88S/A124S A125S	++	9
	W51S/M54S/V82S/W88S/L112A A124S/A125S	+	9
45	W51S/M54S/V82S/W88S/L112A A124S/A125S/L130A	+++	9

	W51S/M54S/V82S/W88S/I119A A124S/A125S	+	9
5	W51S/M54S/V82S/W88S/L112A I119A/A124S/A125S	+++	9
	W51S/M54S/V82S/W88S/A124S A125S/L130A	+++	9
	W51S/V82S/W88S/A125S/A125S L130A	+++	9
10	K52S	++++	3
	M54L	++++	10
	M54S/V56S	++++	9
	W57S/V82S/W88S/L112A/I119A A124S/A125S	+++	9
15	E62A	++++	3
	W64A	++++	3
	Q65A	++++	3
	G66A	++++	3
	L69A	++++	3
20	L69N	++++	4
	S71A	+++	3
	A73G	++++	3
	R76A	++++	3
25	V82S	+/-	9
	V82S/W88S/V144N	++	9
	V82S/W88S/A124S/A125S	++++	9
	N83Q	++	9
	Q92A	++++	3
30	L93A	++++	3
	K97A	++++	3
	S100A	++++	3
	G101A	++++	3
	L102A	++++	8
35	R103A	++	2
	S104A	++	3
	S104N	+/-	6
	L105A	++	8
	L105F	+/-	6
40	T106A	++++	3
	T107A	+++	8
	L108A	++	3
	L109A	+++	8
	L112A	+/-	9
45	L112A/I119S/L130A/I133A	++++	9
	P122Q	+/-	6
	A124P/A125T	++++	4
	A125T	++++	4

	A125N/A127S	++++	4
	L130A	+/-	9
	D136A	++++	3
	R139A	++++	3
5	K140A	++++	3
	R143A	++++	3
	S146A	++++	3
	N147A	++++	3
	R150A	+++	3
10	K152A	++	3
	L153A	+++	3
	K154A	++++	3
	L155A	++++	3
	Y156A	++	3
15	T157A	++++	3
	G158A	++++	3
	E159A	++++	3
	R162K/T163D/G164E/D165L	++	3
	R162H/T163H/G164H/D165H/		
20	R166H/ (167) H	++++	3
	 E2-5	++	3
	E13-17	++++	2
	E32-36	++	3
25	E43-47	++	3
	E53-57	++	3
	E78-82	+	3
	E111-119	+++	3
	E115-121	+++	2
30	E120-122	++++	5
	E123-125	++++	5
	E126-129	+++	5
	E163-166	++++	3
	K116 (insertion of LISEEDL)	++++	3
35			
	# Relative to wildtype is defined as follows:		
	++++	= wildtype or better activity	
	+++	= c.a. 75% of wildtype activity	
40	++	= c.a. 50% of wildtype activity	
	+	= c.a. 25% of wildtype activity	
	+/-	= mutant EPO reported to be active, however, data not complete for assessment of activity relative to wildtype.	
45			

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As used herein, the "natural function" of a polypeptide means its function prior to covalent modification of its N-terminal  $\alpha$ -amino group. Natural functions include, for example, enzymatic activity, receptor binding (e.g. antibodies), ligand binding, and immunogenicity.

The instant methods described more fully below cause the loss of the N-terminal  $\alpha$ -amino group of the polypeptide being covalently modified. Accordingly, the polypeptide must have a primary structure such that its natural function is preserved after covalent modification, and cannot be eliminated. The natural function of the polypeptide is "eliminated" by the removal of its N-terminal  $\alpha$ -amino group if such removal reduces, by more than 99%, the capacity of the polypeptide to perform its natural function. In one embodiment, the removal does not reduce the capacity of the polypeptide to perform its natural function by more than 90%. In the preferred embodiment, the removal does not reduce the capacity of the polypeptide to perform its natural function by more than 50%.

As used herein, a "hydrazone bond" is a bond comprising the covalent structure NH-N=C, an "oxime bond" is a bond comprising the covalent structure O-N=C, a "reduced hydrazone bond" is a bond comprising the covalent structure NH-NH-C, and a "reduced oxime bond" is a bond comprising the covalent structure O-NH-C. Compounds containing reduced hydrazone and oxime bonds are provided herein, since these bonds possess greater chemical stability.

As discussed above, methods are known in the art for binding water-soluble polymers to the N-terminal  $\alpha$ -carbon atom of a polypeptide via a hydrazone bond so long as the N-terminal amino acid residue is serine or threonine. These known methods will not work on polypeptides having

any other N-terminal residue. Although these known methods differ fundamentally from the instant methods, they do result in N-terminal serine and threonine polypeptides having a polymer bound at the N-terminal  $\alpha$ -carbon atom via a hydrazone bond. For this reason, the instant first composition does not encompass a polypeptide bound to a polymer via a hydrazone bond, where the polypeptide's N-terminal amino acid residue is serine or threonine.

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The water-soluble polymers used in the instant invention include, but are not limited to, (a) dextran and dextran derivatives, including dextran sulfate, cross linked dextrin, and carboxymethyl dextrin; (b) cellulose and cellulose derivatives, including methylcellulose and carboxymethyl cellulose; (c) starch and dextrines, and derivatives thereof; (d) polyalkylene glycol and derivatives thereof, including PEG, mPEG, PEG homopolymers, polypropylene glycol homopolymers, copolymers of ethylene glycol with propylene glycol, wherein said homopolymers and copolymers are unsubstituted or substituted at one end with an alkyl group; (e) heparin and fragments of heparin; (f) polyvinyl alcohol and polyvinyl ethyl ethers; (g) polyvinylpyrrolidone; (h) a,b-poly[(2-hydroxyethyl)-DL-aspartamide; and (i) polyoxyethylated polyols. These polymers can be linear, branched, or star-shaped with a wide range of molecular weight. In the preferred embodiment, the polymer is mPEG.

30

When the instant compositions are to be used as pharmaceuticals, the polymer is non-toxic. Furthermore, when a polymer is said to have a given molecular weight, that molecular weight may only be approximate, reflecting the average molecular weight of a population of polymer molecules differing with respect to one another in regard to the number of subunits present in each molecule.

In one embodiment, the PEG or derivative thereof has a molecular weight of from about 700 to about 20,000 daltons. In the preferred embodiment, the PEG or derivative thereof has a molecular weight of about 5,000 daltons. Also, in the preferred embodiment of the instant compositions, the polypeptide is EPO, and the polymer is mPEG having a molecular weight of about 5,000 daltons.

This invention also provides four methods of covalently binding a water-soluble polymer to the N-terminal  $\alpha$ -carbon atom of a polypeptide. The first method, which binds the polymer to the carbon atom via a hydrozone bond, comprises the steps of

(a) contacting the polypeptide with (i) glyoxylate ion or derivative thereof at a concentration of from about 0.1 M to about 2.0 M, (ii) a transition metal ion at a concentration of from about 10  $\mu$ M to about 1 M, and (iii) a Lewis base at a concentration of from about 10 mM to about 10 M, at a pH of from about 3.0 to about 8.0 and a temperature of from about 0°C to about 100°C, so as to form a transaminated

polypeptide having an N-terminal  $\alpha$ -carbonyl group; and

(b) contacting the transaminated polypeptide, at a pH of from about 1.0 to about 7.5, with a water-soluble polymer having a moiety covalently bound thereto which reacts with the transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form a hydrazone bond, thereby covalently binding the polymer to the N-terminal  $\alpha$ -carbon atom of the polypeptide via a hydrazone bond, with the proviso that the polymer has a molecular weight of from about 200 to about 200,000 daltons, and the natural function of the polypeptide is not eliminated upon removal of its N-terminal  $\alpha$ -amino group.

15

The second method, which binds the polymer to the carbon atom via an oxime bond, comprises the steps of

(a) contacting the polypeptide with (i) glyoxylate ion or derivative thereof at a concentration of from about 0.1  $M$  to about 2.0  $M$ , (ii) a transition metal ion at a concentration of from about 10  $\mu M$  to about 1  $M$ , and (iii) a Lewis base at a concentration of from about 10  $mM$  to about 10  $M$ , at a pH of from about 3.0 to about 8.0 and a temperature of from about 0°C to about 100°C, so as to form a transaminated polypeptide having an N-terminal  $\alpha$ -carbonyl group; and

(b) contacting the transaminated polypeptide, at a pH of from about 1.0 to about 7.5, with a water-soluble polymer having a moiety covalently bound thereto

which reacts with the transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form an oxime bond, thereby covalently binding the polymer to the N-terminal  $\alpha$ -carbon atom of the polypeptide via an oxime bond, with the proviso that the polymer has a molecular weight of from about 200 to about 200,000 daltons, and the natural function of the polypeptide is not eliminated upon removal of its N-terminal  $\alpha$ -amino group.

The third method comprises the steps of the first method, as well as a further step of reducing the hydrazone bond formed in step (b). The fourth additional method comprises the steps of the second method, as well as a further step of reducing the oxime bond formed in step (b). The reducing step can be performed by using, for example, sodium borohydride ( $\text{NaBH}_4$ ) and sodium cyanoborohydride ( $\text{NaBH}_3\text{CN}$ ), according to known methods.

Glyoxylate ion derivatives include, but are not limited to, glyoxylamide and phenylglyoxyl ions. Transition metal ions include, but are not limited to, cupric, nickel, cobaltous, or zinc ions. Lewis bases include, but are not limited to, acetate and pyridine.

Moieties which react with the transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form a hydrazone bond include, but are not limited to, hydrazine carboxylate, hydrazine, semicarbazide, hydrazide, thiosemicarbazide, carbonic acid dihydrazide, carbazide, thiocarbazide, and arylhydrazide. Water-soluble polymers

with these moieties covalently bound thereto are commercially available. In addition, moieties which react with the transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form an oxime bond include, but are not limited 5 to, oxylamine. Water-soluble polymers with oxylamine (as well as other oxime-forming moieties) covalently bound thereto are commercially available.

In the preferred embodiment of the instant methods, the 10 pH for step (a) is from about 5.0 to about 7.0, the pH for step (b) is from about 3.0 to about 5.0, and the protein is EPO or derivative thereof.

In one embodiment of the instant methods, the polymer 15 is PEG or derivative thereof. In a further embodiment, the PEG or derivative thereof has a molecular weight of from about 700 to about 20,000 daltons. In the preferred embodiment, the PEG or derivative thereof has a molecular weight of about 5,000 daltons.

20 In one embodiment of the first method, the moiety bound to the polymer which is reacted with the transaminated polypeptide is hydrazine carboxylate. In the preferred embodiment, the polypeptide is EPO, the polymer is mPEG 25 having a molecular weight of about 5,000 daltons, and the moiety covalently bound to the polymer is hydrazine carboxylate.

30 In one embodiment of the second method, the moiety bound to the polymer which is reacted with the transaminated polypeptide is oxylamine.

In each of the instant methods, the preferred contacting time for step (a) is from 20 minutes to 2 hours, and for step (b), the preferred contacting time and temperature are from 10 to 50 hours and from 4°C to room 5 temperature, respectively.

With certain polypeptides, the N-terminus of a polypeptide is "buried", i.e. not exposed to solvents or reagents therein, when the polypeptide is in its native 10 conformation. Reagents such as tetramethylurea or urea may be used to unfold such a polypeptide in order to permit its N-terminal residue to undergo the required reactions of the instant methods.

15 This invention also provides a pharmaceutical composition which comprises an effective amount of the instant first or second composition, and a pharmaceutically acceptable carrier. By way of example, the instant pharmaceutical composition may comprise an 20 amount of the instant mPEG-EPO effective to treat a subject suffering from anemia.

Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited 25 to, 0.01-0.1 M and preferably 0.05 M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene 30 glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers

include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

Finally, this invention provides kits for use in preparing the instant compositions. The first kit, for preparing the first instant composition, comprises the following:

(a) a glyoxylate ion or derivative thereof;  
(b) a transition metal ion;  
(c) a Lewis base; and  
20 (d) a water-soluble polymer having a molecular weight of from about 200 to about 200,000 daltons, and having a moiety covalently bound thereto which reacts with a transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form a hydrazone bond, thereby covalently binding the polymer to 25 the N-terminal  $\alpha$ -carbon atom of the polypeptide.

The second kit, for use in preparing the second instant composition, comprises the following:

(a) a glyoxylate ion or derivative thereof;  
30 (b) a transition metal ion;  
(c) a Lewis base; and

(d) a water-soluble polymer having a molecular weight of from about 200 to about 200,000 daltons, and having a moiety covalently bound thereto which reacts with a transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form an oxime bond, thereby covalently binding the polymer to the N-terminal  $\alpha$ -carbon atom of the polypeptide.

The reagents in these kits may be packaged in a pre-determined quantity, and may be contained in separate compartments. Alternatively, certain reagents may be contained in the same compartment as the constraints of the instant methods permit. Finally, the kits may further comprise reducing reagents for generating reduced hydrazone and oxime bonds according to the instant methods, as well as suitable buffers and reaction vessels.

This invention will be better understood by reference to the Experimental Examples which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Examples1. Preparation of Transaminated EPO

5        5 mg of EPO in 20 mM sodium citrate (pH 6.9) and  
100 mM NaCl was exchanged to 100 mM sodium acetate (pH  
7.0) buffer using Centricon-10 (Amicon, Beverly, MA).  
The final concentrations were adjusted to 1 mg/ml EPO, 2  
M sodium acetate, 0.4 M acetic acid, 0.1 M glyoxylic  
10 acid, and 10 mM cupric sulfate (pH 5.5) (Figure 1). The  
reaction was allowed for 2 hours at room temperature,  
and was stopped by adding 100 ml of 0.5 M EDTA.  
Transaminated EPO was purified via a Sephadex G-25  
15 column (Pharmacia, Piscataway, NJ) using a 100 mM sodium  
acetate (pH 4.5) buffer.

The extent of transamination was estimated by 2,4-dinitrophenylhydrazine as described in the literature (Fields, R. et al., *Biochem. J.*, 1971, 121, 587).  
20 Extinction at 370 nm was measured after the first few minutes and after one hour. The difference in absorbance is proportional to the amount of carbonyl groups present on the EPO molecule. The transaminated EPO was also subjected to amino acid analysis on an ABI 420H system  
25 (Applied Biosystems, Foster City, CA) using pre-column PITC chemistry. The results indicate that lysine residues, i.e. non-N-terminal residues, were not transaminated.

2. Preparation of mPEG-EPO with mPEG-Hydrazine Carboxylate

Transaminated EPO (1 mg) in 100 mM sodium acetate  
5 (pH 4.5) was adjusted to 0.5 M sodium chloride to a final volume of 1ml, to which 10 mg of mPEG5000 hydrazine carboxylate (Shearwater Polymers, Hunstville, AL) was added. The reaction mixture was stirred for 40 hours at room temperature, and purified via a Sephadex 10 S-200 column (Pharmacia, Piscataway, NJ) using a 20 mM sodium citrate (7.0) buffer containing 100 mM NaCl. Additionally, 0.1% SDS may be added to the reaction mixture to increase the conjugation yield.

15 In gel permeation chromatography, the mPEG-EPO conjugate showed a substantially increased molecular weight compared to those of EPO and mPEG5000 hydrazine carboxylate (Figure 2).

20 Matrix-assisted laser desorption mass spectrometry (Finnigan-MAT LaserMAT 2000, linear time-of-flight) was used to characterize mPEG-EPO by molecular weight determination (Figure 3). mPEG5000 hydrazine carboxylate shows an ion at m/z 5157.4. EPO shows a two-charge monomer (m/z 14604), a one-charge monomer (m/z 28569), a dimer (m/z 57208) and a trimer (m/z 85284). Similarly, 25 mPEG-EPO shows a two-charge monomer (m/z 17092), a one-charge monomer (m/z 34279), a dimer (m/z 69071) and a trimer (m/z 102955).

A circular dichroism (CD) spectrum (Jobin-YVON CD6, Dichrograph Spectrometer Instruments, SA, Edison, NJ) of mPEG-EPO showed that the protein retained the  $\alpha$ -helical bundle structure present in native EPO (data not shown).  
5 This result means that a PEG molecule at the N-terminal end of EPO does not disrupt its secondary structure.

3. Preparation of mPEG-EPO with mPEG-hydrazide, mPEG-semicarbazide and oxylamine

10 Transaminated EPO (1 mg) in 100 mM sodium acetate (pH 4.5) was adjusted to 0.5 M sodium chloride, 0.1% SDS to a final volume of 1 ml, and 10-20 mg of mPEG5000 hydrazide, semicarbazide or oxylamine were added. The 15 reaction mixture was stirred for 40 hours at room temperature, and purified via a Sephadryl S-200 column (Pharmacia, Piscataway, NJ) using a 20 mM sodium citrate (7.0) buffer containing 100 mM NaCl.

20 The mPEG-EPO conjugates were analyzed by 4-15% SDS-PAGE (Bio-Rad, Hercules, CA) with various methods: Coomassie stain (specific for proteins), Western blot (specific for EPO), and iodine stain (specific for PEG). The migration distance of higher molecular weight mPEG-25 EPO conjugates on SDS-PAGE is less than that of native EPO. The isoelectric focusing pattern indicates that the isoelectric point (pI) of EPO is not significantly altered upon modification. However, transaminated EPO is slightly more acidic than native EPO and mPEG-EPO.

Since the EPO and mPEG-EPO bands are well separated via SDS-PAGE, this technique can be used to monitor efficiency of the conjugation reaction. It was observed that the conjugation reaction was >95% complete when 5 using mPEG5000-hydrazine carboxylate, whereas the reaction was only about 20% complete when using mPEG5000-hydrazide, mPEG5000-semicarbazide, or mPEG5000-oxylamine. Thus, the hydrazine carboxylate moiety appears to be more reactive towards carbonyl groups than 10 is the hydrazide, semicarbazide, or oxylamine moiety.

#### 4. Reactivity of mPEG-EPO with Anti-EPO Antibody

The antigenicity of mPEG-EPO was studied using a 15 Quantikine™ IVD™ EPO ELISA kit (R&D systems, Minneapolis, MN). The assay consists of a microtiter plate coated with a monoclonal antibody to EPO. EPO or mPEG-EPO is allowed to interact with the coated plate. After washing the plate, a conjugate of anti-EPO 20 polyclonal antibody and horseradish peroxidase is added. After removing excess conjugate, a chromogen is added to the wells and is oxidized by the enzyme reaction to form a blue colored complex. The absorbance of this complex is measured at 450 nm.

25

The results of the ELISA assay for mPEG-EPO with a hydrazone bond formed from hydrazine carboxylate (H<sub>Z</sub>C) and hydrazide (H<sub>Z</sub>) are presented in Figure 5. The data indicate that even one PEG molecule attached at the N- 30 terminus of EPO significantly reduces the affinity of

monoclonal antibody binding to EPO, possibly due to steric hindrance.

5. In vitro Activity of mPEG-EPO

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The *in vitro* biological activity of mPEG-EPO was evaluated by a cell proliferation assay using FDC-P1/HER cells, a murine hematopoietic cell line. The cell line expresses the EPO receptor and is dependent on EPO for growth. After the cells are grown in the absence of EPO for 24 hours, EPO or mPEG-EPO was added to the cells. The cells were incubated for 42 hours, and then tritiated thymidine was added to the cells. After 6 hours, cell growth was determined by the incorporation 15 of thymidine.

The results of the cell proliferation assay for transaminated EPO and mPEG-EPO with hydrazone bonds formed from hydrazine carboxylate (HZC) and hydrazide (HZ) are presented in Figure 6. Transaminated EPO shows full biological activity comparable to native EPO as determined by its ED<sub>50</sub>. The mPEG-EPO samples with hydrazone bonds formed from hydrazine carboxylate (HZC) and hydrazide (HZ) only retain 38.5% and 25% activity, respectively, as determined by their ED<sub>50</sub>. The data indicate that one PEG molecule attached at the N-terminus of EPO significantly reduces the affinity of EPO for its receptor, possibly due to steric hindrance.

6. In vivo Activity of mPEG-EPO

The *in vivo* activity of mPEG-EPO was evaluated by an exhypoxic mouse bioassay (Coates, P.M. et al., 5 *Nature*, 1961, 191, 1065). Murine endogenous red cell formation is suppressed by the polycythemia produced through exposures to reduced pressure. The EPO or mPEG-EPO conjugate is injected at the level of 1 unit/mouse. Iron-59 was administered 48 hours after the EPO or mPEG-10 EPO injection. The iron-59 incorporation, which indicates new red blood cell formation, was measured 48, 72 and 96 hours after administration of EPO or mPEG-EPO.

The results of the exhypoxic mouse bioassay for 15 mPEG-EPO with hydrazone bonds formed with hydrazine carboxylate (HZC), hydrazide (HZ) and semicarbazide (SCZ) are presented in Figure 7. The mPEG-EPO samples show high *in vivo* activity as well as longer activity duration, compared to native EPO. The *in vivo* results 20 indicate that mPEG-EPO samples have longer circulation time *in vivo* and sustained release of EPO during circulation.

7. Preparation of mPEG-Fibrin 17-29 Dimer

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Fibrin 17-29 dimer has the following structure:

Gly-Pro-Arg-Val-Val-Glu-Arg-His-Gln-Ser-Ala-Cys-Lys

S

S

30

Gly-Pro-Arg-Val-Val-Glu-Arg-His-Gln-Ser-Ala-Cys-Lys

2 mg of fibrin 17-29 dimer were dissolved in 0.5 ml of 2 M sodium acetate, 0.4 M acetic acid, 0.1 M glyoxylic acid, and 10 mM cupric sulfate (pH 5.5). The reaction was allowed to proceed for 2 hours at room temperature, and was stopped by adding 20 ml of 0.5 M EDTA. Transaminated fibrin dimer was purified via a Sephadex G-10 column (Pharmacia, Piscataway, NJ) using a 100 mM sodium acetate (pH 4.5) buffer. Transaminated fibrin dimer showed significantly reduced Gly in the amino acid analysis, indicating the transamination of N-terminal Gly.

1 mg of transaminated fibrin dimer in 0.5 ml of 100 mM sodium acetate (pH 4.5) was added to 10 mg of mPEG5000 hydrazine carboxylate. The reaction mixture was stirred for 24 hours at room temperature. The mPEG-fibrin dimer was purified by anion-exchange chromatography with a HEMA IEC BIO CM column (Alltech). Mobile phase A was 20 mM sodium acetate (pH 5.5). Mobile phase B was 0.2 M MOPS, 0.05 M potassium phosphate monobasic and 0.25 M potassium phosphate dibasic (pH 7.5). The gradient was 100% A for 5 minutes, then 0 to 100% B in 25 minutes. A new peak at 14.5 minutes, appearing before unmodified fibrin dimer (18 minutes), was collected for further analysis. A laser desorption mass spectrum showed the ion at m/z 8070.9, which proved that one PEG molecule was attached to the fibrin dimer (m/z 2986.8).

What is claimed is:

1. A composition of matter consisting essentially of a peptide or a protein and a water-soluble polymer covalently bound thereto at the N-terminal  $\alpha$ -carbon atom of the polypeptide, with the proviso that (a) the polymer and polypeptide are bound to each other via a hydrazone bond or reduced hydrazone bond, (b) the polymer has a molecular weight of from about 200 to about 200,000 daltons, (c) the natural function of the polypeptide is not eliminated upon removal of its N-terminal  $\alpha$ -amino group, and (d) the protein's N-terminal amino acid residue is not serine or threonine.
- 15 2. A composition of matter consisting essentially of a polypeptide and a water-soluble polymer covalently bound thereto at the N-terminal  $\alpha$ -carbon atom of the polypeptide, with the proviso that (a) the polymer and polypeptide are bound to each other via an oxime bond or reduced oxime bond, (b) the polymer has a molecular weight of from about 200 to about 200,000 daltons, and (c) the natural function of the polypeptide is not eliminated upon removal of its N-terminal  $\alpha$ -amino group.
- 20 3. The composition of claim 1, wherein the protein is EPO or derivative thereof.
- 25 4. The composition of claim 1 or 2, wherein the polymer is PEG or derivative thereof.

5. The composition of claim 1 or 2, wherein the PEG or derivative thereof has a molecular weight of from about 700 to about 20,000 daltons.
- 5 6. The composition of claim 1 or 2, wherein the PEG or derivative thereof has a molecular weight of about 5,000 daltons.
- 10 7. The composition of claim 1, wherein the protein is EPO, and the polymer is mPEG having a molecular weight of about 5,000 daltons.
- 15 8. A method of covalently binding a water-soluble polymer to the N-terminal  $\alpha$ -carbon atom of a polypeptide, which comprises the steps of
  - (a) contacting the polypeptide with (i) glyoxylate ion or derivative thereof at a concentration of from about 0.1 M to about 2.0 M, (ii) a transition metal ion at a concentration of from about 10  $\mu$ M to about 1 M, and (iii) a Lewis base at a concentration of from about 10 mM to about 10 M, at a pH of from about 3.0 to about 8.0 and a temperature of from about 0°C to about 100°C, so as to form a transaminated polypeptide having an N-terminal  $\alpha$ -carbonyl group; and
  - 20 (b) contacting the transaminated polypeptide, at a pH of from about 1.0 to about 7.5, with a water-soluble polymer having a moiety covalently bound thereto which reacts with the transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form a hydrazone bond, thereby covalently binding the
- 25
- 30

polymer to the N-terminal  $\alpha$ -carbon atom of the polypeptide, with the proviso that the polymer has a molecular weight of from about 200 to about 200,000 daltons, and the natural function of the polypeptide is not eliminated upon removal of its N-terminal  $\alpha$ -amino group.

9. A method of covalently binding a water-soluble polymer to the N-terminal  $\alpha$ -carbon atom of a polypeptide, which comprises the steps of

(a) contacting the polypeptide with (i) glyoxylate ion or derivative thereof at a concentration of from about 0.1 M to about 2.0 M, (ii) a transition metal ion at a concentration of from about 10  $\mu$ M to about 1 M, and (iii) a Lewis base at a concentration of from about 10 mM to about 10 M, at a pH of from about 3.0 to about 8.0 and a temperature of from about 0°C to about 100°C, so as to form a transaminated polypeptide having an N-terminal  $\alpha$ -carbonyl group; and

(b) contacting the transaminated polypeptide, at a pH of from about 1.0 to about 7.5, with a water-soluble polymer having a moiety covalently bound thereto which reacts with the transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form an oxime bond, thereby covalently binding the polymer to the N-terminal  $\alpha$ -carbon atom of the polypeptide, with the proviso that the polymer has a molecular weight of from about 200 to about 200,000 daltons, and the

natural function of the polypeptide is not eliminated upon removal of its N-terminal  $\alpha$ -amino group.

10. The method of claim 8 or 9, wherein the pH for step  
5 (a) is from about 5.0 to about 7.0.
11. The method of claim 8 or 9, wherein the pH for step  
10 (b) is from about 3.0 to about 5.0.
12. The method of claim 8 or 9, wherein the protein is  
15 EPO or derivative thereof.
13. The method of claim 8 or 9, wherein the polymer is  
20 PEG or derivative thereof.
14. The method of claim 13, wherein the PEG or derivative  
25 thereof has a molecular weight of from about 700 to  
about 20,000 daltons.
15. The method of claim 14, wherein the PEG or derivative  
30 thereof has a molecular weight of about 5,000  
daltons.
16. The method of claim 8, wherein the moiety covalently  
25 bound to the polymer is hydrazine carboxylate.
17. The method of claim 8, wherein the protein is EPO,  
the polymer is mPEG having a molecular weight of  
about 5,000 daltons, and the moiety covalently bound  
30 to the polymer is hydrazine carboxylate.

18. The method of claim 9, wherein the moiety covalently bound to the polymer is oxylamine.
19. The method of claim 8, which further comprises the step of reducing the hydrazone bond formed in step (b) so as to form a reduced hydrazone bond.
20. The method of claim 9, which further comprises the step of reducing the oxime bond formed in step (b) so as to form a reduced oxime bond.
21. A pharmaceutical composition which comprises an effective amount of the composition of claim 1 or 2, and a pharmaceutically acceptable carrier.

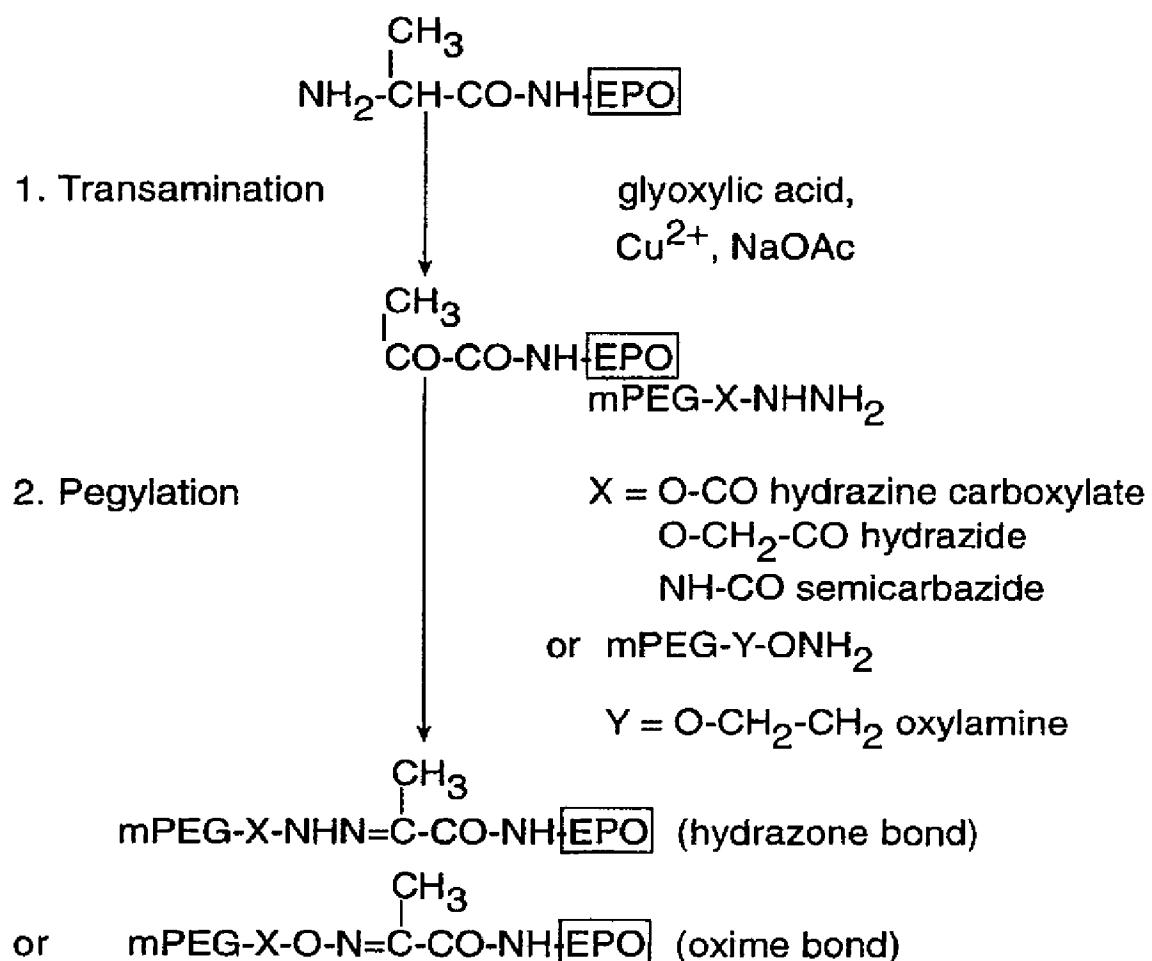
15 22. A kit for use in preparing the composition of claim 1, which comprises the following:

- (a) a glyoxylate ion or derivative thereof;
- (b) a transition metal ion;
- (c) a Lewis base; and
- 20 (d) a water-soluble polymer having a molecular weight of from about 200 to about 200,000 daltons, and having a moiety covalently bound thereto which reacts with a transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form a hydrazone bond, thereby covalently binding the polymer to the N-terminal  $\alpha$ -carbon atom of the polypeptide.

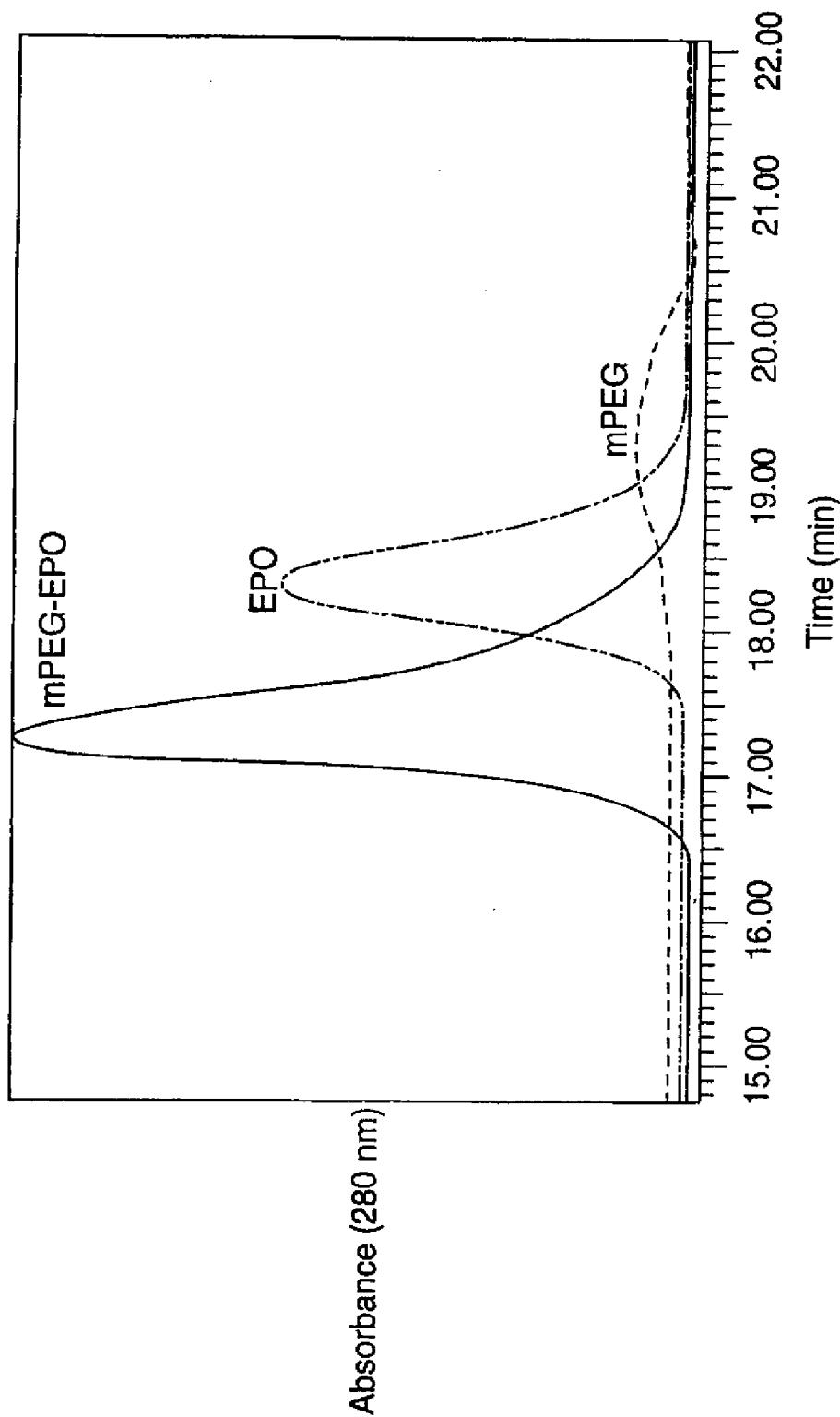
25

23. A kit for use in preparing the composition of claim 2, which comprises the following:
- (a) a glyoxylate ion or derivative thereof;
- (b) a transition metal ion;

(c) a Lewis base; and  
(d) a water-soluble polymer having a molecular weight of from about 200 to about 200,000 daltons, and having a moiety covalently bound thereto which reacts with a transaminated polypeptide's N-terminal  $\alpha$ -carbonyl group to form an oxime bond, thereby covalently binding the polymer to the N-terminal  $\alpha$ -carbon atom of the polypeptide.

**FIG. 1**

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**FIG. 2**

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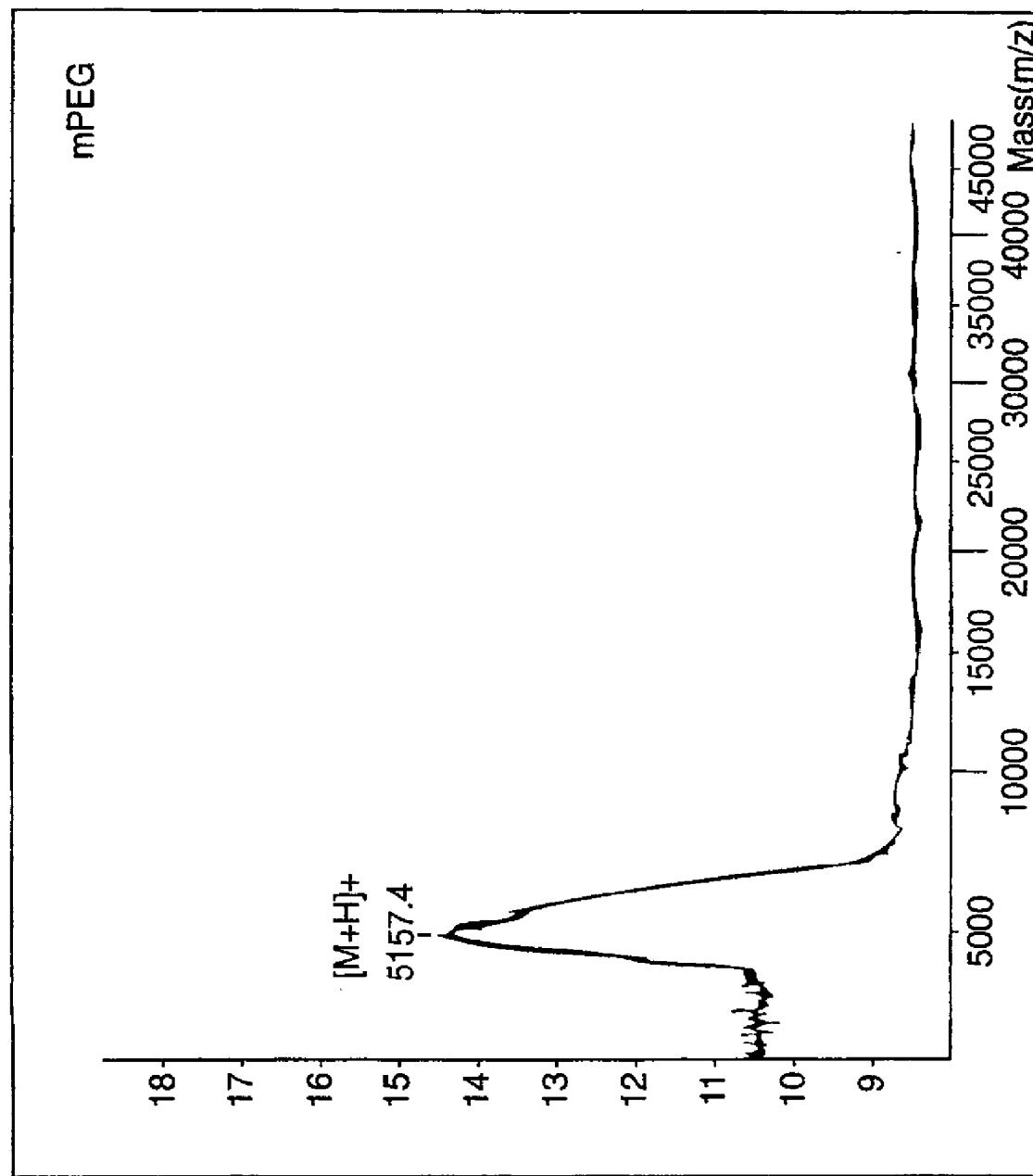


FIG. 3A

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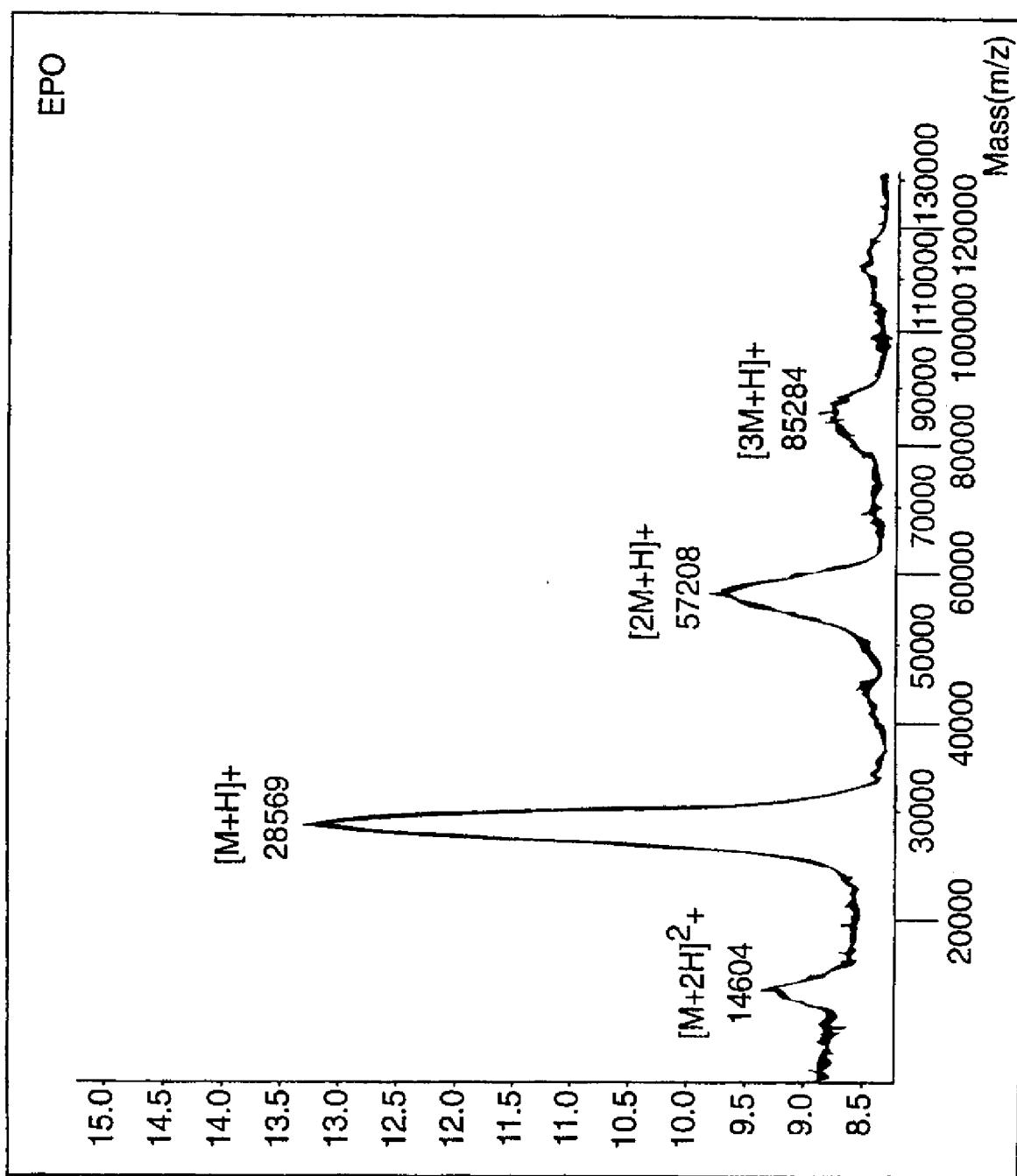


FIG. 3B

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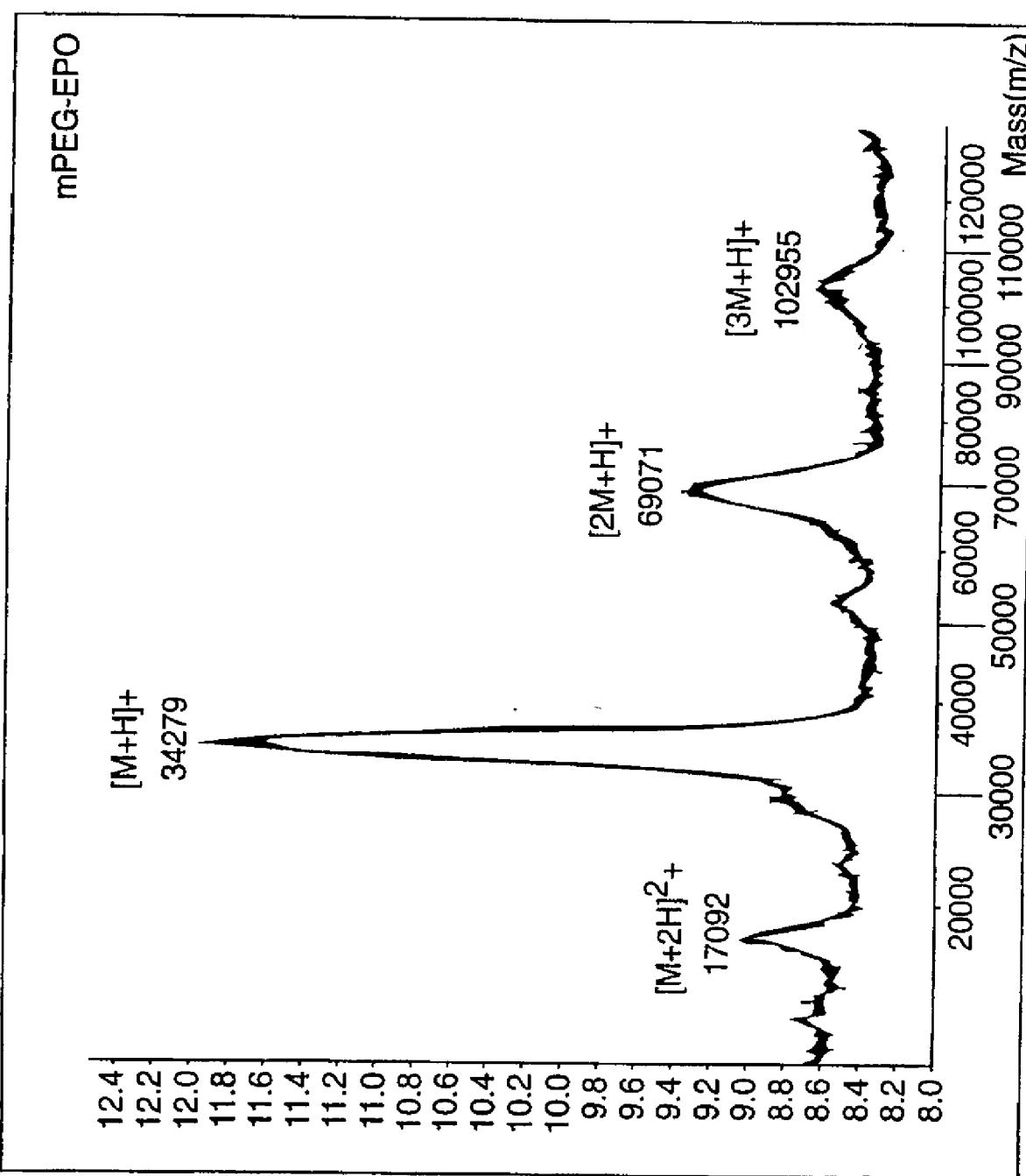
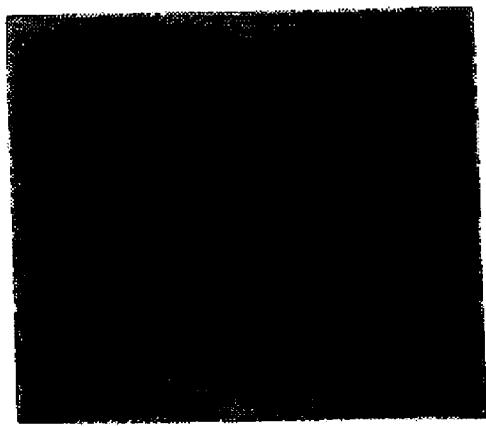


FIG. 3C

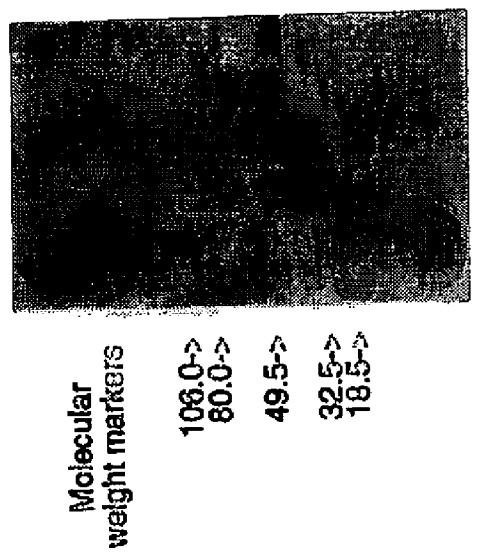
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**FIG. 4A**

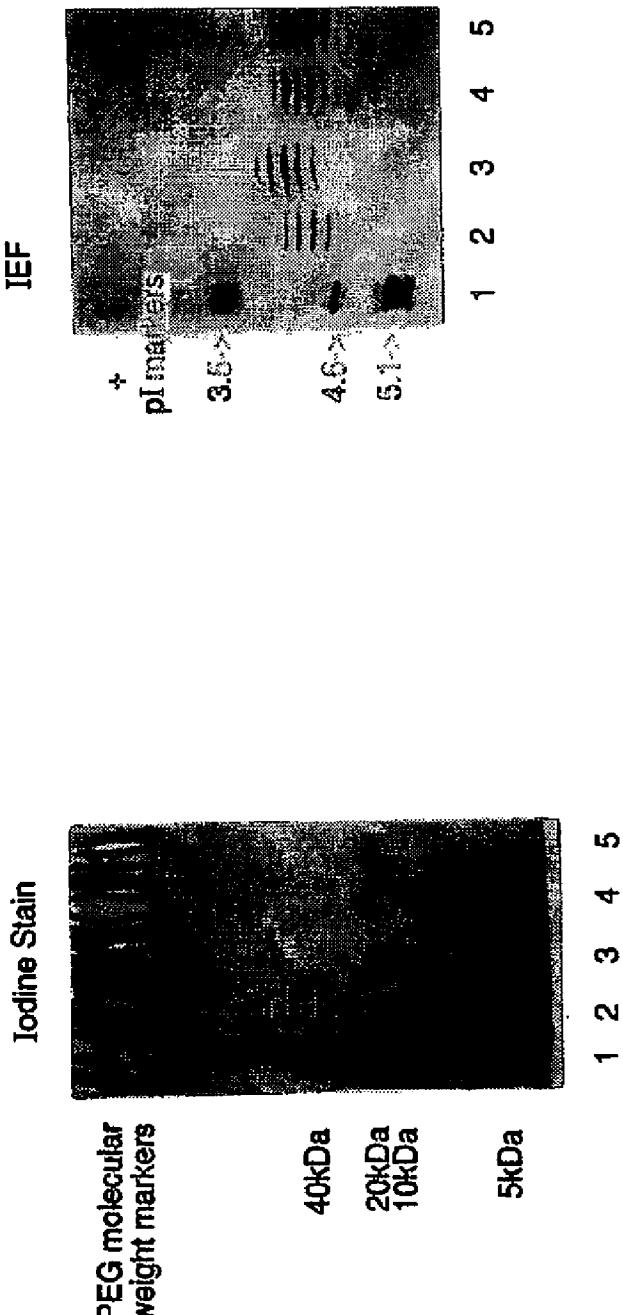
Coomassie Stain

**FIG. 4B**

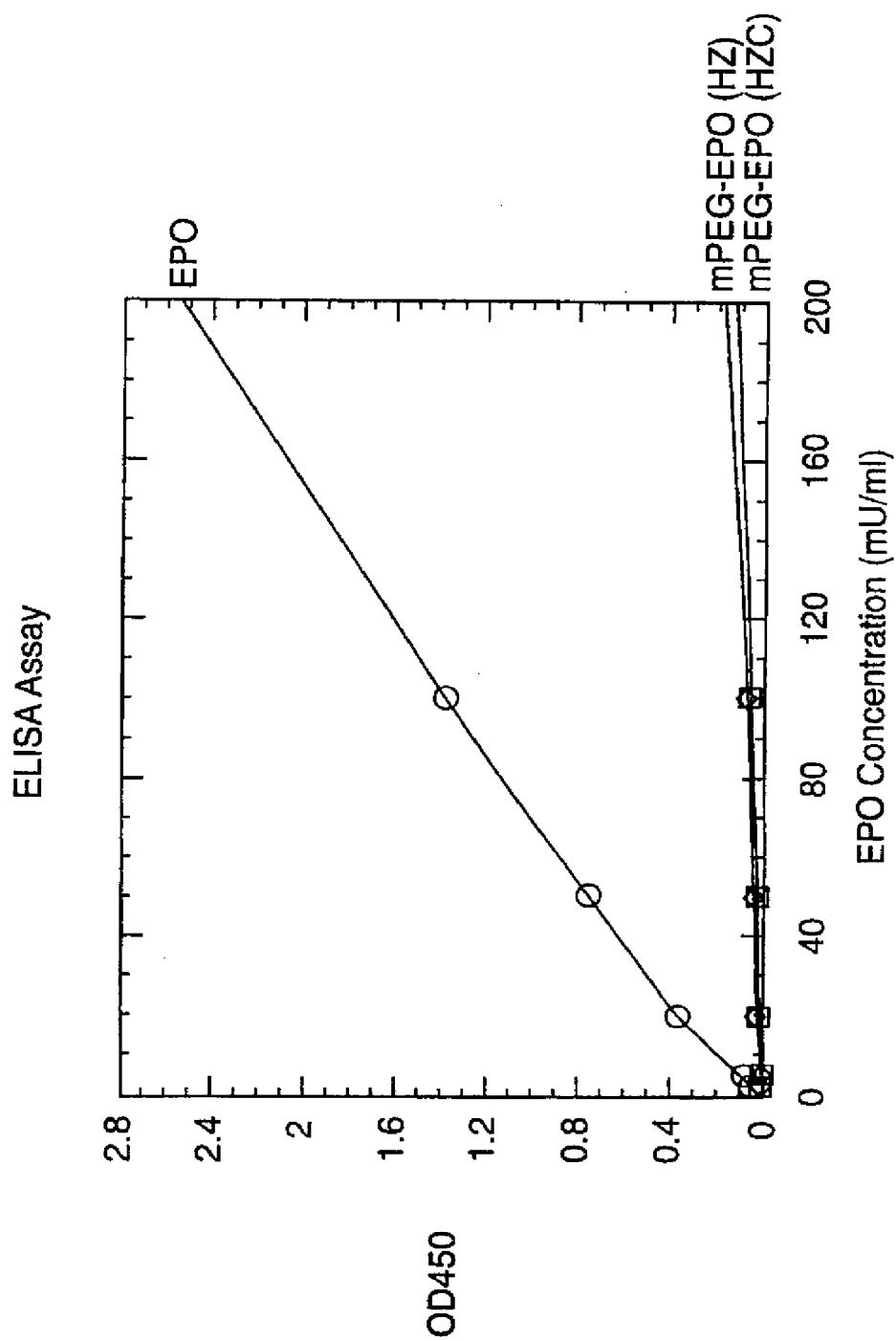
Western Blot



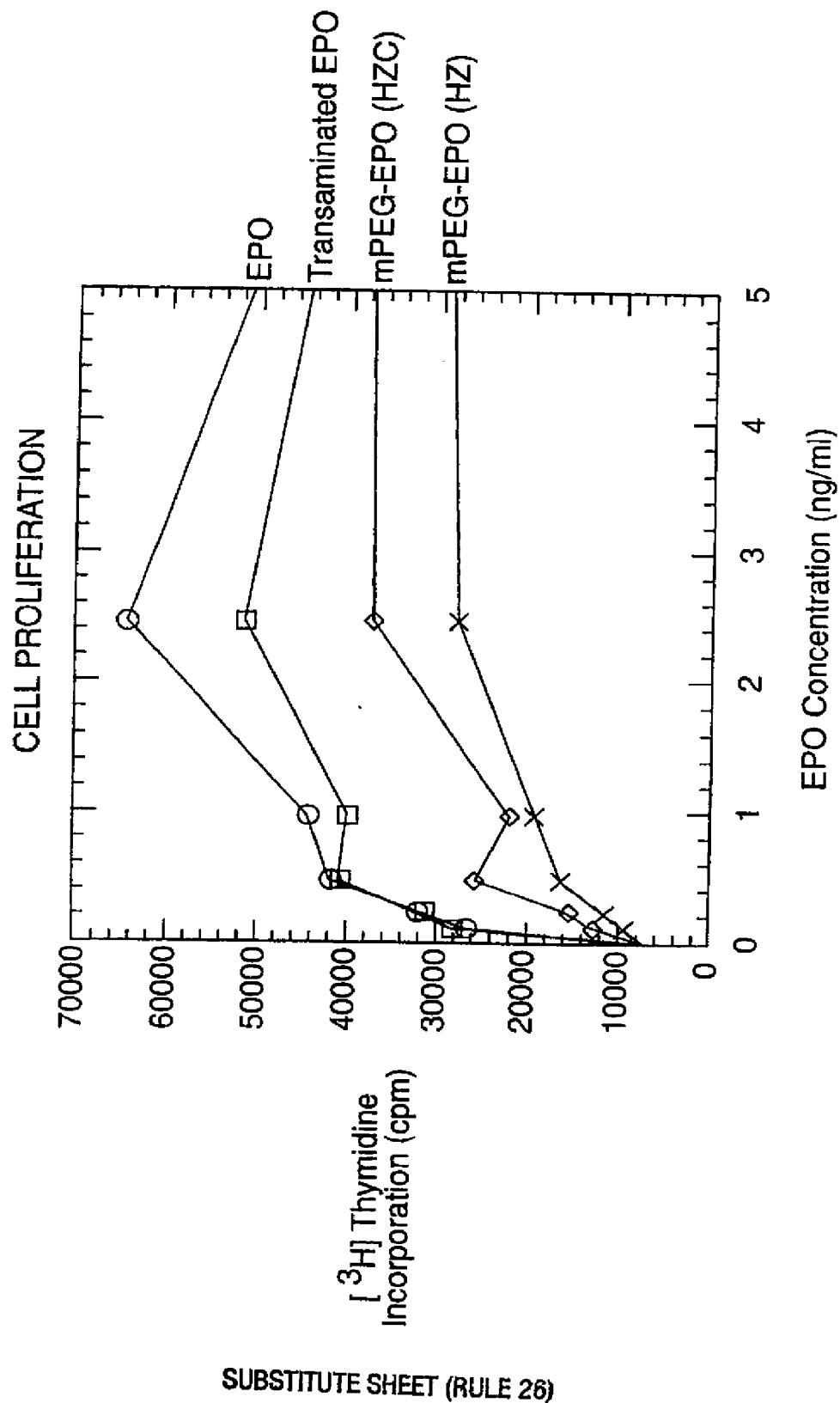
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**FIG. 4C**

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**FIG. 5**

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**FIG. 6**

SUBSTITUTE SHEET (RULE 26)

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**FIG. 7**